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Modulation of *Bacillus subtilis* Levansucrase Gene Expression by Sucrose and Regulation of the Steady-State mRNA Level by *sacU* and *sacQ* Genes

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In *Bacillus subtilis*, the extracellular enzyme levansucrase is synthesized in the presence of sucrose. A termination structure between the transcription start site and the structural gene was the apparent site for regulation by sucrose of transcription into the structural gene. Sequence analysis of the *sacB* leader region from two strains constitutive for levansucrase synthesis showed a single base change in the stem of this termination structure. This single base change also led to the constitutive synthesis of a *sacB'-'lacZ* fusion, whereas the wild-type *sacB'-'lacZ* fusion was induced by the addition of sucrose. S1 nuclease mapping of *sacB* transcripts with probes labeled either within the termination structure or 3' to the termination structure showed that sucrose did not increase the number of transcripts extending into the termination structure; however, sucrose did increase the number of transcripts extending past the termination structure. Two pleiotropic mutations which affect the expression of levansucrase, *sacQ36* hyperproducing [*sacQ36*(Hy)] and *sacU32*(Hy), were separately introduced into the strain carrying the *sacB'-'lacZ* fusion. These mutations each increased the expression levels of the *sacB'-'lacZ* fusion. S1 mapping showed increased levels of transcript initiating at the *sacB* promoter in strains with the *sacQ36*(Hy) and *sacU32*(Hy) mutations. This increased transcription appeared to be independent of the sucrose-regulated transcription termination, suggesting the existence of at least two different mechanisms for the regulation of *sacB* expression.

Sucrose metabolism of *Bacillus subtilis* has been studied extensively (15, 16). Two saccharolytic enzymes, sucrase and levansucrase, can be detected in crude extracts of *B. subtilis* 168 after induction by sucrose. Sucrase is an intracellular enzyme, whereas levansucrase is secreted. Both enzymes act as β-fructofuranosidases, and levansucrase also catalyzes the formation of the high-molecular-weight fructose polymer levan. The structural gene of levansucrase, *sacB*, maps between *cysB* and *hisA* on the *B. subtilis* chromosome (17). Several types of regulatory mutations that affect levansucrase expression have been identified. One class of mutations, termed *sacR* constitutive (*sacR*^{c}), is very tightly linked to the *sacB* locus and leads to constitutive synthesis of levansucrase (15). The intracellular sucrase remains normally inducible by sucrose in strains with *sacR*^{c} mutations (16). A second regulatory locus, *sacS*, affects the synthesis of both sucrase and levansucrase (15, 16). Three types of mutations have been defined at this locus: *sacS*^{c}, which leads to constitutive synthesis of both enzymes; *sacS*$^-$, which is defective for the synthesis of levansucrase; and *sacS* hyperproducing [*sacS*(Hy)], which leads to hyperproduction of levansucrase. Two other mutations, *sacQ36*(Hy) and *sacU32*(Hy), show increased levels of levansucrase which remain inducible by sucrose. The *sacQ*(Hy) and *sacU*(Hy) mutations confer pleiotropic phenotypes and also affect the synthesis of several other extracellular enzymes (14).

The structural gene of levansucrase has been cloned into phage λ by Gay et al. (12). Recently, the nucleotide sequence of the *sacB* gene and its flanking region was determined (11, 29). Between the probable promoter and the structural gene of *sacB* is a potential termination structure (29). A recent report showed that deletions of this termination structure, or single base changes that destabilize the structure, lead to constitutive synthesis of levansucrase (28). A short open reading frame (ORF) extends through this termination structure, and it has been postulated that translation of this ORF might modulate the expression of the *sacB* gene by an attenuation mechanism (29).

In the *B. subtilis trp* operon there is a terminator preceding the *trpE* structural gene that is the site of transcription termination regulated by tryptophan (24). This regulation is by a novel form of attenuation in which a regulatory factor appears to interact directly with the nascent transcript to regulate termination; translation does not appear to be involved in this attenuation (23). The apparent termination structure preceding the *sacB* gene suggested that *sacB* expression might be regulated in a manner similar to that of the *trp* operon.

In this study, we have defined the transcription start point and shown that it precedes the potential termination structure. Analysis of transcripts showed constitutive transcription from the *sacB* promoter; however, these transcripts did not extend into the *sacB* structural gene in the absence of sucrose. Sequence analysis of two mutations that lead to constitutive synthesis of levansucrase showed a single base change in the potential termination structure. These data point to this structure as the site of regulation. Fusions of the ORF in this region to the *lacZ* gene did not show detectable levels of β-galactosidase, indicating that translation of this ORF does not occur, and this ORF must not be involved in the regulation of *sacB*. Analysis of transcripts in strains carrying the *sacU32*(Hy) and *sacQ36*(Hy) showed increased steady-state levels of transcripts from the *sacB* promoter. The effect of these hyperproducing mutations appeared to be separate from the sucrose regulation. We interpret these results by a model of sucrose-regulated attenuation from the

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TABLE 1. Bacterial strains

Strain	Genotype[a]	Source
W168	Prototroph	J. A. Hoch
BG62	*sacR37*[c] *sacA321 sacU32*(Hy) *thr-5*	Pavia University (PB5010)
1A164	*sacR47*[c]	BGSC[b] (1A164)
1A165	*sacU32*(Hy) *trpC2*	BGSC (1A165)
BG129	*sacS32*[c] *trpC2 ery-1*	BGSC (1A88)
BG3019	*sacU32*(Hy) *trpC2*	Spontaneous motile derivative of 1A165
BG29	*sacQ36*(Hy) *trpC2 ald*	Pavia University (PB1713)
BG125	*trpC2 hisA1 thr-5*	J. A. Hoch (QB917)
BG4021	*amyE*::(*sacBp sacB'-'lacZ cat*)	pLEVBGI tf[c] to W168
BG4022	*amyE*::(*sacBp sacR37*[c] *sacB'-'lacZ cat*)	pLEVBG-*sacR* tf to W168
BG4023	*trpC2 sacS32*[c] *amyE*::(*sacBp sacB'-'lacZ cat*) *ery-1*	pLEVBGI tf to BG129
BG4024	*trpC2 thr-5 hisA1 amyE*::(*sacBp sacB'-'lacZ cat*)	pLEVBGI tf to BG125
BG4025	*trpC2 thr-5 sacU32*(Hy) *amyE*::(*sacBp sacB'-'lacZ cat*)	BG3019 td[d] to BG4024
BG4026	*trpC2 hisA1 sacQ36*(Hy) *amyE*::(*sacBp sacB'-'lacZ cat*)	BG29 td to BG4024
BG4027	*trpC2 thr-5 hisA1 amyE*::(*sacBp sacORF-1-'lacZ cat*)	pLEVBG-*sac*ORF-1 tf to BG125
BG4029	*trpC2 thr-5 hisA1 amyE*::(*sacBp sacR37*[c] *sacB'-'lacZ cat*)	pLEVBG-*sacR* tf to BG125
BG4030	*trpC2 thr-5 sacU32*(Hy) *amyE*::(*sacBp sacR37*[c] *sacB'-'lacZ cat*)	BG3019 td to BG4029
BG4031	*trpC2 hisA1 sacQ36*(Hy) *amyE*::(*sacBp sacR37*[c] *sacB'-'lacZ cat*)	BG29 td to BG4029
BG4034	*amyE*::(*sacBp* (−263 to +199)[e] *sacR37*[c] *sacB'-'lacZ cat*)	pLEVBG-*Sau*3A tf to W168
BG4048	*trpC2 hisA1 sacQ36*(Hy)	BG29 td to BG125
BG4049	*trpC2 thr-5 sacU32*(Hy)	BG3019 td to BG125

[a] *cat* indicates the pC194 chloramphenicol acetyltransferase gene.
[b] *Bacillus* Genetic Stock Center, Ohio State University.
[c] tf, Transformation.
[d] td, Transduction.
[e] The numbers correspond to the positions in Fig. 2.

sacB promoter and a separate regulation of the steady-state level of transcripts by the *sacU* and *sacQ* genes.

MATERIALS AND METHODS

Strains, phage, and plasmids. The *B. subtilis* strains used in this study are listed in Table 1. Amylase production was tested by growing colonies overnight on a nutrient broth plate containing 1% (wt/vol) starch and then staining the plate with a solution containing 0.5% (wt/vol) iodine and 1.0% (wt/vol) potassium iodine. The presence of secreted proteases was determined by growth on L broth-skim milk-agar plates containing 1.5% (wt/vol) skim milk (Difco Laboratories). Colonies were tested for β-galactosidase expression on minimal medium plates (26) containing 0.5% glucose and 50 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. *Escherichia coli* MM294 (F⁻ *supE44 endA1 thi-1 hsdR4*) was used as a host for plasmid constructions. *E. coli* JM101 (Δ*lacpro supE thi* F' *traD36 proAB lacI*[q]*Z* Δ*M15*) was used as a host for M13mp11 (19). The λ Charon 4A library of *B. subtilis* chromosomal DNA was provided by E. Ferrari (9). Plasmids pBR325 (6), pUC13 (19), pUC18 (35), and ptrpBGI (23) were used for the constructions of the derivative plasmids described here.

β-Galactosidase assays. *B. subtilis* cells containing the *lacZ* fusions were grown in minimal-CH medium containing 0.05% (wt/vol) Casamino Acids (Difco Laboratories), 0.5% (wt/vol) glucose, and minimal salts (26) supplemented with 5 μg of chloramphenicol per ml. Cells were harvested at an optical density at 600 nm of 0.6 by centrifugation for 3 min in an Eppendorf microfuge and then stored at −20°C. The frozen cells were suspended in 1 ml of Z buffer (20) and then incubated at 37°C for 5 min with 100 μg of lysozyme per ml. After the addition of 0.1% (vol/vol) Triton X-100, samples were assayed for β-galactosidase activity by the method of Miller (20). Total cellular protein was measured with the Bio-Rad Laboratories (Richmond, Calif.) protein assay reagent.

DNA manipulations. The procedures used for isolation of plasmid DNAs, screening of colonies, and phage plaques with ^{32}P-labeled probes were carried out as described before (24, 33). DNA sequencing was conducted by the dideoxy chain termination method of Sanger et al. (21). Certain DNA regions were sequenced by using deoxyadenosine 5'-α-[^{35}S] thio-triphosphate (>600 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) (5). Oligonucleotides (*sacB* probe, 5'-GCACTGCTGGCAGGAGGCGCA-3'; *Hin*dIII primer; 5'-GTTGCTTGTTTTGCAAGCTTTTTGATGTTC-3'; *Acc*I-*Hin*dIII linkers, 5' ATACGGCA-3' and 5' AGCTTGCCGT-3') were provided by the Genentech Organic Synthesis Group. Blot hybridization analysis was performed by the method of Southern (25). Restriction enzymes, polynucleotide kinase, T4 ligase, and S1 nuclease were purchased from commercial sources and used according to the recommendations of the suppliers. Oligonucleotide mutagenesis was performed by a published procedure (36).

S1 nuclease mapping experiment. The restriction fragment containing the *sacB* promoter-leader region was obtained from the plasmid pUCLEVH3-*Sau*3A, which contains the *sacB* 5'-flanking region (*Sau*3A to *Hin*dIII) in pUC13. After pUCLEVH3-*Sau*3A was digested with *Acc*I or *Rsa*I, the 5' end was ^{32}P labeled by polynucleotide kinase-mediated reaction with [γ-^{32}P]ATP (>5,000 Ci/mmol; Amersham) and then cleaved with *Eco*RI. The *sacB* promoter-leader fragments (the region from positions −263 to +70 or −263 to +176) which had been ^{32}P labeled at the 5' ends of the *Acc*I or *Rsa*I site were isolated by polyacrylamide gel electrophoresis and used as hybridization probes. *B. subtilis* strains were grown in 50 ml of minimal-CH medium in the presence or absence of 2% (wt/vol) sucrose. At an optical density at 600 nm of approximately 0.6, cells were harvested, and total cellular RNA was prepared as described before (24). The hybridization probe described above and 5 μg of the bacterial RNA were hybridized and digested with 500 U of S1 nuclease (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) by the method of Aiba et al. (1) and

electrophoresed on a 7% polyacrylamide–8 M urea gel. Size standards were created by G- and C-specific cleavage reactions (18) with the same DNA fragment.

Bacterial transformation and transduction. *E. coli* transformation was performed by the calcium shock procedure (8). Transformants were selected on LB plates supplemented with ampicillin at 50 μg/ml or chloramphenicol at 12.5 μg/ml. *B. subtilis* was transformed by a published procedure (3), except that the cells were suspended in a 2-fold volume of the stage II medium instead of a 10-fold volume. Transformants were selected on Tryptose blood agar base (Difco Laboratories, Detroit, Mich.) plates supplemented with chloramphenicol at 5 μg/ml. PBS1 phage lysate preparation and transduction were performed as described before (13). PBS1 lysates from strains BG3019 and BG29 were kindly provided by Mark Ruppen.

RESULTS

Cloning and sequencing of the *sacB* gene. The λ Charon 4A library of *B. subtilis* chromosomal DNA constructed by Ferrari et al. (9) was used to isolate a DNA fragment containing the *sacB* gene. An oligonucleotide (*sacB* probe) that corresponds to the published DNA sequence (11) of the *sacB* gene was used as a hybridization probe. Five λ clones that were positive by plaque hybridization were obtained. Restriction enzyme analysis with *Eco*RI revealed common 6.0-, 3.4-, and 2.1-kilobase (kb) bands (Fig. 1). The 3.4-kb *Eco*RI fragment, which was reported to contain the *sacB* 5′-flanking region (11), was subcloned into the *Eco*RI site of pBR325, and this plasmid was designated pLEV3.4 (Fig. 1). The nucleotide sequence of the 5′-flanking and amino-terminal region of the *sacB* gene was determined (Fig. 2). This sequence matched that recently reported by Steinmetz et al. (29). One interesting feature of this sequence is that the *sacB* coding region is preceded by a region of dyad symmetry followed by a T-rich sequence (Fig. 2) which resembles ρ-independent transcriptional terminators of *E. coli*.

S1 nuclease mapping of *sacB* transcripts. To determine the transcriptional initiation site of the *sacB* promoter and to determine whether the region of dyad symmetry regulates transcription, a series of S1 nuclease mapping experiments was performed. The first probe was a 333-base-pair (bp) fragment extending from the *Sau*3A site at position −263 to the *Acc*I site at position +70. This probe was labeled with ^{32}P at the 5′ terminus of its *Acc*I site. The second probe was a 439-bp fragment extending from the *Sau*3A site at position −263 to an *Rsa*I site at position +176, labeled at the 5′ terminus of the *Rsa*I site. RNA was extracted from *B. subtilis* grown in minimal-CH medium with glucose or sucrose as the carbon source. Each probe was hybridized to the RNA samples and subsequently digested with S1 nuclease. After denaturation, the length of the protected DNAs was determined. With the probe labeled at the *Acc*I site, the same protected fragment was detected with RNA derived from cultures with and without sucrose (Fig. 3A, lanes 3 and 4). Comparison of the protected fragment with the G and C sequencing tracks suggests that transcription initiates at or near the guanine residue assigned as +1 in Fig. 2. This transcriptional initiation site is located 199 bp before the coding region and is preceded by a potential promoter sequence (−10 region TAGAAT; −35 region TTGCAA) which is typical of the sequences recognized by *B. subtilis* σ43-type RNA polymerase. A different result was seen with the probe labeled at the *Rsa*I site. Only the RNA extracted from cells grown in the presence of sucrose protected a labeled fragment (Fig. 3B, lane 1). This protected fragment

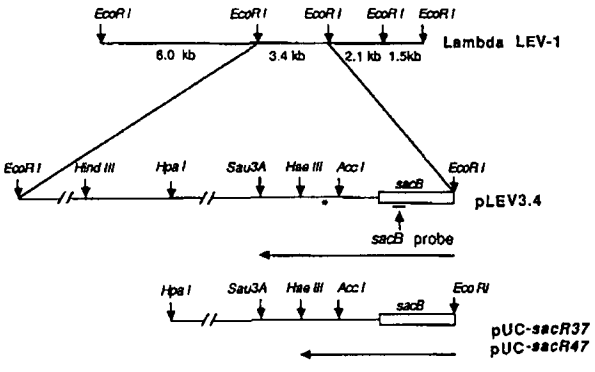

FIG. 1. Inserts of λ LEV-1, pLEV3.4, and pUC-*sacR37*^{c} and pUC-*sacR47*^{c}. The vectors are not depicted; they are λ Charon 4A for LEV-1, pBR325 for pLEV3.4, and pUC18 for pUC-*sacR37* and pUC-*sacR47*. The coding sequence for the levansucrase gene is indicated by double lines. The approximate position of the *sacB* transcription start site is indicated by an asterisk. The extent of the sequenced regions from pLEV3.4, pUC-*sacR37*, and pUC-*sacR47* is indicated by arrows.

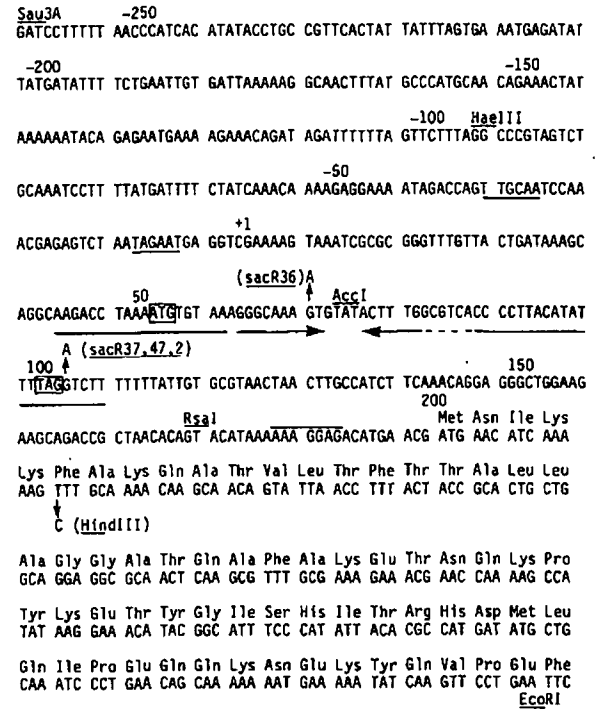

FIG. 2. Nucleotide sequence of the *sacB* promoter-leader region. The sequence is numbered relative to the mRNA start site. The sequences constituting the probable −10 and −35 regions of the *sacB* promoter are underlined. The potential ribosome binding site of the *sacB* gene is overlined. The dyad symmetry structure discussed in the text is indicated by arrows. A downward arrow points to the single base change introduced by M13 mutagenesis to create a *Hin*dIII site, whereas upward arrows point to the single base changes found in the *sacR*^{c} mutants. The initiation and termination codons of the potential open reading frame discussed in the text are boxed.

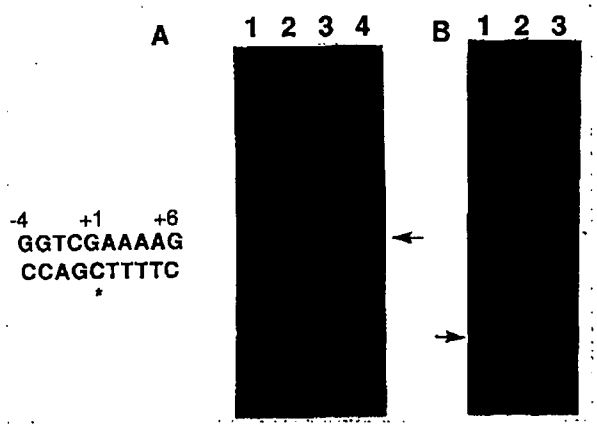

FIG. 3. S1 mapping analysis of the *sacB* promoter. A *Sau*3A (position −263)-to-*Acc*I (+70) DNA fragment was labeled with ^{32}P at the *Acc*I site and used as the probe. This probe was hybridized to 5 μg of total cellular RNA derived from strain BG125, which had been grown in minimal-CH medium with glucose (lane 3) or the same medium with 2% sucrose as the carbon source (lane 4). Lanes 1 and 2 are the C- or G-specific chemical degradation products of the labeled fragment. The sequence surrounding the initiation site is shown to the left. In interpreting this data we have subtracted 2 bases from the size of S1 nuclease-protected bands, since the fragments generated by the chemical sequencing reactions migrate faster than the corresponding fragments generated by S1 nuclease digestion. An arrow points to the S1 nuclease-protected bands. (B) Demonstration of readthrough transcripts in the presence of sucrose. A *Sau*3A (−263)-to-RsaI (+176) DNA fragment was labeled with ^{32}P at the *Rsa*I site and used as the probe. Lanes: 1, total cellular RNA from BG4049 grown in minimal-CH medium with sucrose; 2, RNA derived from BG4049 grown in minimal-CH medium with glucose; 3, G-specific chemical degradation products of the same probe.

showed the same transcription start site as that seen with the probe labeled at the *Acc*I site. These results suggest that transcription from the *sacB* promoter is constitutive, but that only in the presence of sucrose does transcription extend past the termination structure to the *Rsa*I site (Fig. 2). The amount of transcript extending to the *Rsa*I site in the absence of sucrose (Fig. 3B, lane 2) was too low to quantitate by excising and counting the band; however, longer exposures of films gave a rough estimation of at least a 100-fold difference in the transcript levels in the presence and absence of sucrose (data not shown). The S1 analysis gave the same results when mRNA was extracted from BG125 or from strain BG4049 [*sacU32*(Hy)].

Sequence determination of *sacR* mutations. *sacR*^{c} mutations are closely linked to the *sacB* locus (15). Recently Steinmetz et al. (29) localized the *sacR2*^{c} mutation to the region between the *Sau*3A and *Eco*RI sites at positions −263 and +398 by using DNA-mediated transformation. This region was isolated from the *sacR37*^{c} and *sacR47*^{c} mutant strains to determine the nature of these mutations. Chromosomal DNA of BG62 (*sacR37*^{c}) or 1A164 (*sacR47*^{c}) strains were digested with *Eco*RI and *Hin*dIII, and DNA fragments approximately 2 kb in size were isolated from an acrylamide gel. The DNA fragments obtained were ligated with pUC18 vector that had been digested with *Eco*RI and *Hin*dIII, and the ligation products were transformed into *E. coli* MM294. Ampicillin-resistant colonies were screened by colony hybridization with the synthetic *sacB* probe described above. Several positive colonies from both *sacR37*^{c} and *sacR47*^{c} DNAs were chosen, and their plasmids were isolated. These plasmids were digested with *Eco*RI and *Hin*dIII, and each revealed the expected 2.1-kb fragment. Two plasmids, designated pUC-*sacR37* and pUC-*sacR47*, were derived from *sacR37* and *sacR47* DNA, respectively. The inserts from each plasmid were transferred into appropriate M13 vectors, and the nucleotide sequences were determined from positions +398 to −94 (Fig. 1 and 2). The same single base change from the wild-type sequence was found at position +102 (Fig. 2) in both pUC-*sacR37* and pUC-*sacR47*. This base change is located in the stem of the potential termination structure (Fig. 2).

Construction of a *sacB'-'lacZ* fusion. A *lacZ* fusion was constructed in which the *sacB* promoter and its regulatory region controls hybrid *sacB'-'lacZ* enzyme formation. We had constructed a *lacZ* fusion plasmid, ptrpBGI, that was designed to introduce a single copy of a *trpE'-'lacZ* fusion into the *B. subtilis* chromosome (23). The *trp* promoter in the ptrpBGI plasmid is bounded by unique *Eco*RI and *Hin*dIII sites that can be used as convenient sites for the cloning of the other promoter fragments. A *Hin*dIII site in the correct reading frame was introduced after the fifth codon of the levansucrase gene by oligonucleotide-directed mutagenesis (Fig. 2). Then a 1.3-kb *Hpa*I-*Hin*dIII fragment was subcloned into pUC13 at the *Hin*cII and *Hin*dIII sites, creating the plasmid pUCLEVH3 and providing a convenient *Eco*RI site 35 bp upstream of the former *Hpa*I site. The resultant 1.3-kb *Eco*RI-*Hin*dIII fragment was used to replace the *Eco*RI-*Hin*dIII fragment of ptrpBGI (23), creating the plasmid pLEVBGI (Fig. 4 and 5). In this construction, the *sacB* promoter-leader region, ribosome-binding site, and first five codons of the *sacB* gene were fused in correct reading frame to the eighth codon of the *lacZ* gene (7). This

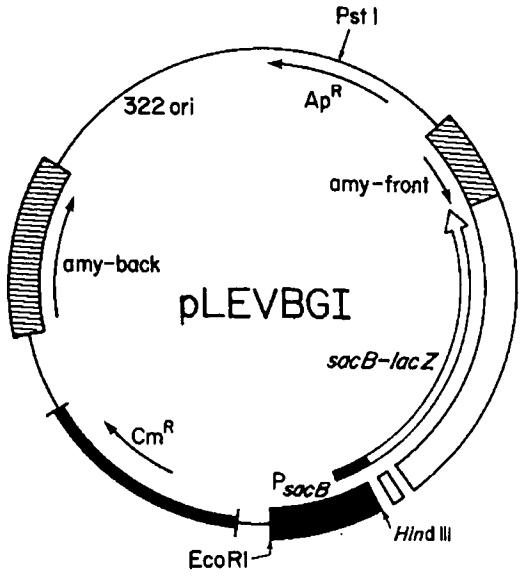

FIG. 4. Structure of pLEVBGI. The 1.3-kb *Eco*RI-*Hin*dIII fragment containing the *sacB* promoter-leader region and first 5 codons of the structural gene was obtained from pUCLEVH3. The direction of transcription of the amylase gene (amy), ampicillin resistance (Ap^R) gene, chloramphenicol resistance (Cm^R) gene, and *sacB'-'lacZ* fusion are indicated by arrows. 322 ori indicates the replication origin of pBR322.

TABLE 2. *sacB'-'lacZ* hybrid β-galactosidase expression with different integrated plasmids

Strain	Integrated plasmid	Relative sp act of β-galactosidase[a]		
		Glucose	Sucrose	Glucose plus sucrose
BG4021	pLEVBGI	≤0.1	1.0[b]	1.2
BG4034	pLEVBG-*Sau*3A	≤0.1	1.1	ND[c]
BG4022	pLEVBG-*sacR*	16.2	16.1	16.0
BG4027	pLEVBG-*sacORF-1*	≤0.1	≤0.1	ND
W168	None	≤0.1	≤0.1	≤0.1

[a] The assay of each culture was carried out in triplicate, except BG4027 which was done in duplicate. Cells were grown in minimal-CH medium with 2% sucrose or 0.5% glucose or both as the carbon sources (indicated in subheadings).

[b] The induced specific activity in BG4021 (average, 4.3 Miller units per mg of total cellular protein) was set at 1.0.

[c] ND, Not determined.

sacB'-'lacZ fusion and a chloramphenicol-resistant determinant were flanked by portions of the *B. subtilis* amylase gene (Fig. 4) to introduce it into the amylase gene region of the *B. subtilis* chromosome. The pLEVBGI plasmid was linearized by digestion with *Pst*I and transformed into strain W168 by selection for chloramphenicol resistance. Thirty chloramphenicol-resistant transformants were patched onto a starch plate; all of them showed an amylase-minus phenotype, indicating that a portion of the plasmid was integrated into the amylase gene. The 30 transformants were also picked onto 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside plates with or without sucrose. All colonies were pale blue in the presence of sucrose and white when glucose was used as the sole carbon source. One transformant (BG4021) was picked, and the proper single copy integration of the *sacB'-'lacZ* fusion was confirmed by Southern blot analysis with the amylase and *lacZ* gene fragments as probes (data not shown). BG4021 was grown in a minimal-CH medium in the presence or absence of sucrose, and the levels of β-galactosidase were measured. In minimal-CH medium with 2% sucrose as the carbon source, there were approximately 4 U of β-galactosidase per mg of protein, whereas in the minimal-CH medium with 0.5% glucose there was less than 1 U/mg (Table 2). When this strain was grown in the presence of both 0.5% glucose and 2% sucrose, we detected virtually the same levels of β-galactosidase as the strain grown in the presence of only sucrose, suggesting that this fusion was induced by sucrose but not repressed by glucose.

The pLEVBGI plasmid contains approximately 1.1 kb of DNA upstream of the *sacB* coding region. A second *sacB'-'lacZ* fusion plasmid, pLEVBG-*Sau*3A (Fig. 5), was constructed which contains the *sacB* promoter region from

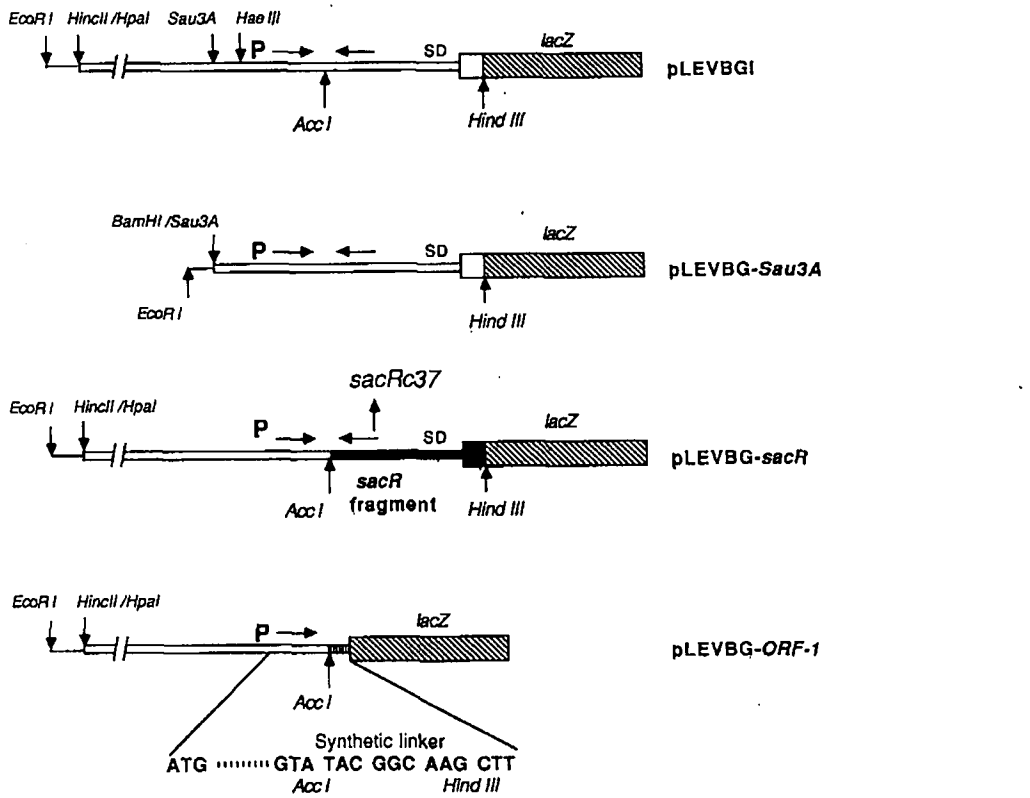

FIG. 5. Structures of the integrated portions of plasmid pLEVBGI, pLEVBG-*Sau*3A, pLEVBG-*sacR*, and pLEVBG-*ORF-1*. In each case the *lacZ* fusion is oriented in the plasmid vector as indicated in Fig. 4 for pLEVBGI. The single lines at the left of each insert are the *Eco*RI-*Hin*cII and *Eco*RI-*Bam*HI linkers derived from pUC13. The open double lines indicate fragments derived from pUCLEVH3, the solid double lines indicate fragments derived from p*UCsacR37*, and the hatched double lines indicate the *lacZ* coding region. *Acc*I-*Hin*dIII synthetic linkers were used to create a translational fusion to the *lacZ* gene in pLEVBG-*ORF-1*. The nucleotide sequence of the *sacORF1-lacZ* fusion was confirmed. The approximate position of the region of dyad symmetry discussed in the text is indicated by horizontal arrows. P and SD indicate *sacB* promoters and Shine-Dalgarno regions, respectively. DNA segments are not shown to scale.

TABLE 3. *sacB'-'lacZ* hybrid β-galactosidase expression in wild-type and mutant strains

Strain	Relevant genotype	Integrated plasmid	Relative sp act of β-galactosidase[a]	
			Sucrose	Glucose
BG4024		pLEVBGI	1.0[b]	≤0.1
BG4023	*sacS32*[c]	pLEVBGI	4.3	2.9
BG4025	*sacU32*(Hy)	pLEVBGI	532	1.6
BG4026	*sacQ36*(Hy)	pLEVBGI	33	≤0.1
BG125		None	≤0.1	≤0.1

[a] The assay of each culture was carried out in triplicate. Cells were grown in minimal-CH medium with 2% sucrose or 0.5% glucose as the carbon source (indicated in subheadings).

[b] The induced specific activity in BG4024 (average, 5.0 Miller units per mg of total cellular protein) was set at 1.0.

positions −263 to +199. This plasmid was integrated into the *B. subtilis* chromosome as described above, and the β-galactosidase activity was measured after growth in minimal-CH medium with glucose or sucrose as the carbon source. The levels of β-galactosidase in the strain carrying pLEVBG-*Sau*3A were virtually identical with those of the strain carrying pLEVBGI and were controlled by sucrose in the medium (Table 2). These results established that the 462 bp preceding the structural gene (region from positions −263 to +199 in Fig. 2) contain the information necessary for expression and regulation of the *sacB* gene.

Construction of a *sacB'-'lacZ* fusion with the *sacR37*[c] mutation. To determine whether the single base change in *sacR37*[c] leads to constitutive synthesis of levansucrase, we constructed a *sacB'-'lacZ* fusion that contains this mutation. First, oligonucleotide mutagenesis was performed to create a *Hin*dIII site at the fifth and sixth codons of the *sacB* gene fragment obtained from the *sacR37*[c] mutant. The 142-bp *Acc*I-*Hin*dIII fragment derived from the *sacR37*[c] mutant was isolated and used to replace the wild-type *Acc*I-*Hin*dIII fragment of pLEVBGI (Fig. 5). This plasmid, pLEVBG-*sacR*, was introduced into the amylase gene region of the *B. subtilis* chromosome as described above, and proper single-copy integration was confirmed by Southern blot hybridization. Strain BG4022 carrying pLEVBG-*sacR* showed constitutive expression of the hybrid β-galactosidase in both the presence and absence of sucrose in the medium (Table 2).

Construction of a *lacZ* fusion with the open reading frame in the *sacB* leader region. Steinmetz et al. (29) pointed out the presence of a short ORF in the leader region of the *sacB* gene. To determine whether this potential ORF is actually translated in vivo, we constructed a *lacZ* fusion in which eight codons of this ORF were fused in the correct reading frame to the *lacZ* gene (Fig. 5). This *lacZ* fusion was introduced into the *B. subtilis* chromosome, and the transformant (BG4027) was assayed for β-galactosidase synthesis. BG4027 showed no detectable β-galactosidase activity above background levels (Table 2). Prolonged incubation of strain BG4027 on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside plates also showed no detectable β-galactosidase activity. This transformant showed an amylase-negative phenotype, and the presence of the *lacZ* fusion in the chromosome was also confirmed by Southern blot hybridization (data not shown). These results indicate that this ORF is not translated in vivo.

Analysis of the *sacB'-'lacZ* fusion in strains carrying *sacS*[c], *sacQ*(Hy), or *sacU*(Hy) mutations. To determine the effect of a *sacS*[c] mutation on the expression levels of the *sacB'-'lacZ* fusion, the plasmid pLEVBGI was linearized and introduced into the chromosome of strain BG129 (*sacS32*[c]). Strain BG4023 [*sacS32*, *amy*E::(*sacB'-'lacZ*)] was grown in minimal-CH medium in the presence and absence of sucrose and assayed for β-galactosidase activity. Strain BG4023 showed constitutive expression of β-galactosidase both in the presence and absence of sucrose (Table 3), although the expression level was lower in the absence of sucrose.

Two other classes of mutations, *sacQ*(Hy) and *sacU*(Hy), that lead to the hyperproduction of the levansucrase have been described (14, 15). To analyze the effect of these mutations on the *sacB'-'lacZ* fusion, the pLEVBGI plasmid was first transformed into the strain BG125 to create strain BG4024 [*hisA1 thr-5 trpC2 amyE::(sacB'-'lacZ)*]. PBS1 lysates from the strain BG3019 [*sacU32*(Hy)] or BG29 [*sacQ36*(Hy)] were prepared and separately transduced into the strain BG4024. The *sacU32*(Hy) and *sacQ36*(Hy) mutations are linked in PBS1 transduction with *hisA* and *thr-5*, respectively, and after selection for His^+ or Thr^+ the introduction of these mutations could be easily determined by the hyperproduction of proteases on LB-skim milk plates. Strain BG4026 [*sacQ36 amyE::(sacB'-'lacZ)*] showed 30-fold higher activity of β-galactosidase in the presence of sucrose than that of the parental strain, BG4024, and strain BG4025 [*sacU32 amyE::(sacB'-'lacZ)*] showed 500-fold higher activity of β-galactosidase in the presence of sucrose (Table 3). In both strains the β-galactosidase activity was inducible by sucrose. As a control, the *sacU32*(Hy) and *sacQ36*(Hy) mutations were introduced into strain BG125 (the parental strain of the BG4024). Strains BG4048 and BG4049 showed no detectable β-galactosidase activity above background level (data not shown).

Introduction of the pLEVBG-*sacR* plasmid into *sacQ*(Hy) and *sacU*(Hy) mutant strains. To test the effect of the *sacR* lesion on the *sacQ*(Hy) and *sacU*(Hy) mutations, they were introduced by PBS1 transduction into a strain carrying the *sacR37*[c] derivative of the *sacB'-'lacZ* fusion. The *sacQ*(Hy) or *sacU*(Hy) strains carrying the integrated pLEVBG-*sacR* showed high levels of β-galactosidase expression in both the presence and absence of sucrose, whereas the BG4029 (*sacU*$^+$ *sacQ*$^+$) strain carrying the same pLEVBG-*sacR* showed lower levels of constitutive expression (Table 4).

Determination of *sacB* transcript levels in *sacU*(Hy) and *sacQ*(Hy) mutant strains. An S1 mapping experiment was performed with RNAs derived from the *sacU*(Hy) and *sacQ*(Hy) strains. First, the *sacU32* and *sacQ36* markers were transduced into strain BG125 by PBS1-mediated transduction to construct strains with similar genetic backgrounds. The parental strain BG125 and the derivative strains BG4049 (*sacU32*) and BG4048 (*sacQ36*) were grown in minimal-CH medium with or without sucrose as the carbon source, and total cellular RNAs were prepared from

TABLE 4. Effects of *sacR* lesion on *sacB'-'lacZ* expression in *sacQ*(Hy) and *sacU*(Hy) mutants

Strain	Relevant genotype	Integrated plasmid	Relative sp act of β-galactosidase[a]	
			Sucrose	Glucose
BG4024		pLEVBGI	1.0[b]	≤0.1
BG4029		pLEVBG-*sacR*	18	17
BG4031	*sacQ36*(Hy)	pLEVBG-*sacR*	54	37
BG4030	*sacU32*(Hy)	pLEVBG-*sacR*	574	329

[a] The assay of each culture was carried out in triplicate. Cells are grown in the minimal-CH medium with 2% sucrose or 0.5% glucose as the carbon source (indicated in subheadings).

[b] The induced specific activity in BG4024 was set at 1.0.

each strain. The previously described DNA probe labeled at its 5′ *Acc*I site was hybridized to 5 μg of each RNA and digested with S1 nuclease. When RNAs from BG125 grown in the presence or absence of sucrose were used, the same 70-nucleotide protected bands, which corresponded to the transcript initiated around position +1, were observed (Fig. 6, lanes 1 and 2). When RNA was derived from BG4049 (*sacU32*), an increased amount of the protected bands was observed in both the presence and absence of sucrose (Fig. 6, lanes 3 and 4). Strain BG4048 (*sacQ36*) also showed increased levels of transcript (Fig. 6, lanes 5 and 6). The relative ratios of these bands were determined both by densitometric scanning of the autoradiographs and by excising these bands from the gel and determining radioactivity. In a typical experiment, approximately 20- to 70-fold and 6- to 10-fold increases were observed in the protected bands from the *sacU* and *sacQ* strains, respectively, relative to the parental strain.

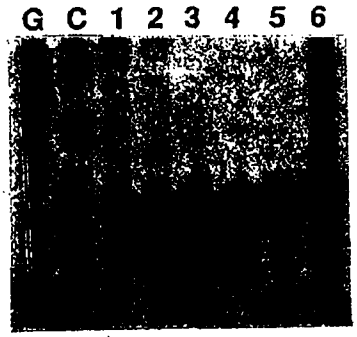

FIG. 6. S1 mapping experiment with RNAs prepared from *sacU32*(Hy) and *sacQ36*(Hy) mutant strains. The [^{32}P]DNA probe used in Fig. 3 labeled at the 5′ terminus of the *Acc*I site was hybridized to total cellular RNAs of *B. subtilis* BG125, BG4049, and BG4048 and treated with S1 nuclease. The G- or C-specific degradation reactions with the same DNA fragment provide size markers. The total cellular RNA was prepared from the cells grown in minimal-CH medium with glucose or with sucrose. Samples (5 μg) of RNA and DNA probes (approximately 5 × 10^6 cpm/μg) were hybridized as described in Materials and Methods. Lanes: 1, RNA from BG125 grown with 0.5% glucose; 2, RNA from BG125 grown with 2% sucrose; 3, RNA from BG4049 [*sacU32*(Hy)] grown with 0.5% glucose; 4, RNA from BG4049 grown with 2% sucrose; 5, RNA from BG4048 [*sacQ36*(Hy)] grown with 0.5% glucose; 6 RNA from BG4048 grown with 2% sucrose.

DISCUSSION

In recent reports, Steinmetz et al. (28, 29) suggested that the terminator in the *sacB* leader region was involved in the regulation of the *sacB* gene. DNA sequence determination of two constitutive mutations, *sacR2*^{c} (G to A at position 67 in Fig. 2) and *sacR36*^{c} (G to A at position 102 in Fig. 2), showed that both had changes within the stem of the termination structure that would lower its stability (28). Further, in vitro-derived deletions of the termination structure led to constitutive synthesis of levansucrase (28). On the basis of this evidence it was postulated that transcription of the *sacB* gene might be controlled by a regulated transcription termination event in a manner analogous to that of the *B. subtilis trp* operon (22–24). The data presented in this paper support the hypothesis that sucrose regulates the expression of the *sacB* gene by increasing transcription past the transcription terminator preceding the *sacB* gene. In addition, the amount of transcript initiating at the *sacB* promoter can be influenced by the *sacU* and *sacQ* genes.

S1 mapping of the *sacB* promoter defined the transcription start site 199 bp upstream of the *sacB* structural gene. Thus, transcription from the *sacB* promoter must extend past the potential termination structure at positions +41 to +106 in the leader region to reach the *sacB* structural gene. In the presence or absence of sucrose, the same start point for transcription was utilized (Fig. 3A and 6), and the same amount of transcript which extended to the *Acc*I site at position +70 was detected. However, only in the presence of sucrose was a significant amount of transcript detected which extended past the terminator to the *Rsa*I site at +176 (Fig. 3B). Although the precise site of this termination event has not been determined, it probably occurs at the termination structure. The finding that the *sacR*^{c} mutations, which lead to constitutive synthesis of levansucrase, are single nucleotide changes in the termination structure supports the argument that this structure is necessary for sucrose regulation. Since there is no evidence for sucrose effects on the level of transcription initiation, these *sacR*^{c} mutations cannot be operator mutations. Although each of the four sequenced *sacR*^{c} mutations lowers the stability of the termination structure (the predicted ΔG for the wild type is −39.6 kcal [ca. 165.7 kJ]/mol, whereas the ΔG for the *sacR36* mutant is −35.2 kcal [ca. 147.3 kJ]/mol, and the ΔG for the *sacR2*, *sacR37*, and *sacR47* mutants is −30.4 kcal [ca. 128.0 kJ]/mol) (30), they each appear to make a very stable hairpin structure; it is not clear why they would no longer function as terminators.

In the *E. coli trp* operon, translation of a short leader peptide modulates the formation of alternative secondary structures in the *trp* leader region (34). There is a short ORF in the *sacB* leader region which overlaps the termination structure. However, when this ORF was fused to the β-galactosidase gene, no β-galactosidase activity could be detected above background levels in a strain carrying this fusion. This indicates that this ORF is not translated in vivo and probably is not involved in the regulation of *sacB* transcription.

The regulation of the expression of the *sacB* gene appears to be similar to that of the *B. subtilis trp* operon (24). The *B. subtilis trp* promoter is also constitutive, a termination structure is present, and transcription past the termination structure is modulated by the formation of alternative secondary structures in the nascent transcript (24). We have proposed a model in which a tryptophan-activated regulatory factor binds to the nascent transcript to modulate the alternative secondary structures (24). No alternative secondary structure is apparent in the *sacB* leader region. Our current model for *sacB* regulation is that a regulatory molecule, activated by sucrose, binds at or near the stem region of the RNA termination structure of the nascent transcript, preventing formation of the terminator and allowing transcription of the *sacB* gene. A candidate for such a regulatory molecule is the product of the *sacS* gene (15, 16). When the *sacB′-′lacZ* fusion was introduced into a strain with *sacS*^{c} mutation, the resulting strain showed constitutive expression of β-galactosidase, implying that the *sacS* is a *trans*-acting regulatory molecule. Mutations localized to *sacS* have three phenotypes (16), which could be explained as follows. Constitutive mutations could be those which bind to the nascent transcript in the absence of sucrose, preventing the

formation of the terminator. Negative mutations could be those in which the regulatory molecule is no longer synthesized or cannot bind to the transcript; thus all transcripts would terminate. Hyperproducing mutations could be explained as overproducers of the regulatory molecule, especially if it is present in limiting amounts. There are suggestions that this might be the situation, since sucrose does not induce the *sacB'-'lacZ* fusion to as high a level as that seen with the various constitutive mutations.

Similar models can be proposed which are consistent with a model of negative regulation. We favor the positive model mainly due to the lack of an alternative secondary structure to prevent termination. In vitro transcription should be useful to test these models, since positive regulation predicts that termination will occur in the absence of the positive regulatory molecule. Another model which has not been excluded is that of differential degradation. The S1 analysis of transcripts could also be explained if the *sacB* transcripts were protected from degradation in the presence of sucrose (or actively degraded in the absence of sucrose).

Genetic analysis of sucrose metabolism in *B. subtilis* led to the identification of pleiotropic mutations, *sacU*(Hy) and *sacQ*(Hy), which affect the synthesis of not only levansucrase but also a number of other secreted gene products (14, 15). Since all of the identified gene products affected by these pleiotropic mutations are secreted, it seemed logical that these mutations affected the process of secretion (4). A report from Aubert et al. (4) indicated that the *sacU*(Hy) mutation did not increase the levansucrase mRNA level as determined by RNA-dot blot analysis, suggesting that its effect was translational or posttranslational. However, our findings contradict this report. Our S1 mapping analysis shows a significant increase in transcripts from the *sacB* promoter in both *sacU*(Hy) and *sacQ*(Hy) strains. There is a stimulation of 20- to 70-fold by the *sacU*(Hy) mutation and 6- to 10-fold by the *sacQ*(Hy) mutation. The introduction of the *sacU*(Hy) and *sacQ*(Hy) mutations into a strain carrying the *sacB'-'lacZ* fusion led to large increases in β-galactosidase activity. These fusion proteins have only the first five amino acids of *sacB*, are cytoplasmic, and presumably do not interact with the secretory machinery of the cell.

Our findings are supported by a recent report in which the first eight amino acids of the subtilisin (*aprA*) gene were fused to the *lacZ* gene and the *sacU*(Hy) mutation was introduced into a strain carrying an integrated *aprA-lacZ* fusion (10). The introduction of the *sacU*(Hy) mutation increased the expression of the *aprA-lacZ* about fusion sevenfold (10). The identical fusion also showed increased levels in a strain carrying the *sacQ*(Hy) mutation (D. J. Henner and M. Yang, unpublished data). Although it has not been determined whether transcription of the *aprA-lacZ* fusion is increased by these mutations, these data suggest that the effect of the *sacQ*(Hy) and *sacU*(Hy) mutations on the expression of both the *aprA* and *sacB* gene products occurs at a step before the secretion process.

At present we do not know how the *sacU*(Hy) and *sacQ*(Hy) mutations act to increase the expression of these genes. The increased steady-state level of transcripts detected in the S1 analyses could be due to either increased transcription from the *sacB* promoter or an increased half-life. It is also not clear whether the *sacU* and *sacQ* genes act independently and whether their effects are direct or indirect. Four of the genes known to be affected by these mutations have been sequenced: those encoding α-amylase, levansucrase, neutral protease, and subtilisin (27, 29, 32, 33). There are no obvious similarities in their promoter or leader regions that might be target sites for a common regulatory factor. Recent experiments with a *lacZ* fusion to the intracellular serine protease of *B. subtilis* have shown that synthesis of this gene is also increased by *sacU*(Hy) and *sacQ*(Hy) mutations (M. Ruppen, personal communication); thus, at least one gene whose product is not secreted is affected by these genes. The only apparent common feature to the genes affected by *sacU* and *sacQ* is that their products are degradative.

The *sacQ* gene product of *B. subtilis* has been identified as a 46-amino-acid polypeptide (31). The *sacQ36*(Hy) mutation has been defined as a single base change which lies near the promoter for the *sacQ* gene (31). This *sacQ36* mutation appears to act by increasing the amount of the 46-amino-acid polypeptide. The *sacU* gene product has also been cloned, and its gene product has been tentatively identified as a 46-kilodalton membrane protein (4). The nature of the change in *sacU*⁻ and *sacU*(Hy) mutations has not been determined, nor has the sequence of the *sacU* gene product been obtained.

After submission of this paper, a report by Aymérich et al. (2) was published which showed that a *sacU*(Hy) mutation increases the steady-state level of *sacB* mRNA and also increases the expression of a *sacB'-'lacZ* fusion.

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